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ALEX1 suppresses colony formation ability of human colorectal carcinoma cell lines

Hiroyoshi Iseki,[1,2,3] Akihiko Takeda,[1,2,6] Toshiwo Andoh,[2,3] Koki Kuwabara,[2,4] Norio Takahashi,[2] Igor V. Kurochkin,[5] Hideyuki Ishida,[4] Yasushi Okazaki[3,6] and Isamu Koyama[1,2]

[1]Department of Digestive Surgery, Saitama International Medical Center, Saitama Medical University, Saitama; [2]Project Research Division, Saitama Medical University, Saitama; [3]Division of Functional Genomics and Systems Medicine, Research Center for Genomic Medicine, Saitama Medical University, Saitama; [4]Department of Gastrointestinal and General Surgery, Saitama Medical Center, Saitama Medical University, Kawagoe, Japan; [5]Genome and Gene Expression Data Analysis Division, Bioinformatics Institute, Matrix, Singapore

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Arm protein lost in epithelial cancers, on chromosome X (*ALEX*; also known as armadillo repeat containing, X-linked [*ARMCX*]) is a novel subgroup within the armadillo (ARM) family, which has several ARM repeat domains. The biological function of classical ARM family members such as β-catenin is well understood, but that of the *ALEX*/*ARMCX* family members is largely unknown. Here we evaluate the effects of *ALEX1* overexpression on *in vitro* colony formation ability and expression of *ALEX1* mRNA in human colorectal tumor. Overexpression of *ALEX1* suppressed the anchorage-dependent and -independent colony formation of human colorectal carcinoma cell lines by the study of stable clones of HCT116 cells expressing *ALEX1* protein. Bisulfite genomic sequencing revealed that the promoter region of *ALEX1* gene was highly methylated in both HCT116 and SW480 cells in comparison with PANC-1 and MCF-7 cells, which express endogenous *ALEX1* mRNA, indicating the capability of promoter methylation to silence *ALEX1* gene in HCT116 and SW480 cells. Our current findings suggest that overexpression of *ALEX1* play a negative role in human colorectal tumorigenesis. (*Cancer Sci* 2012; 103: 1267–1271)

Armadillo (ARM) repeat proteins, characterized by the presence of a repeating 42 amino acid motif, form a large family implicated in a variety of processes such as cell adhesion, embryogenesis and tumorigenesis.[1,2] Armadillo repeats mediate protein–protein interaction with diverse binding partners involved in cell junction assembly, nuclear transport and transcription activation.[3–5] β-Catenin (*CTNND1* gene), a classical member of ARM family, is a multifunctional protein that plays essential roles both at adherence junctions and in Wnt signaling through interaction with E-cadherin and T cell factor/lymphoid enhancer factor (*TCF/LEF*) family transcription factors, respectively.[6,7] Tumor suppressor adenomatous polyposis coli (*APC*) is also an ARM family member and acts synergistically with casein kinase I, glycogen synthase kinase-3β and AXIN to regulate Wnt signaling via β-catenin degradation.[8–10] Approximately 80% of the sporadic colorectal carcinomas have inactivating mutations in *APC* and degradation-resistant mutations in β-catenin occur in around 50% of the remaining colorectal carcinomas. These mutations can cause aberrant nuclear accumulation of β-catenin, leading to the transcriptional activation of the Wnt target genes such as oncogenic c-*MYC* and *CCND1*.[8,9,11–13]

Arm protein lost in epithelial cancers, on chromosome X (*ALEX*; also known as armadillo repeat containing, X-linked [*ARMCX*]) is a novel subgroup within the ARM family. The *ALEX/ARMCX* gene family consists of six genes including three predicted genes (*ALEX1-6*).[14–16] Bioinformatics analyses suggest that the *ALEX1*, *ALEX2* and *ALEX3* are each encoded by a single exon and contain an N-terminal transmembrane domain, some ARM repeat domains, and a DUF634 (domain of unknown function 634).[14–16] However, little is known about the *ALEX/ARMCX* genes. The only report regarding the biological function of the *ALEX/ARMCX* proteins demonstrates that the *ALEX3* directly interacts with the sex determining region Y (Sry)-box 10 (SOX10) transcription factor via the ARM repeat domains and alters its subcellular localization and transcriptional activity.[15] In addition, gene expression analysis revealed that both *ALEX1* and *ALEX2* mRNA is expressed in a variety of adult human tissues, including colon, but dramatically reduced or even undetectable in several human carcinoma cell lines and tissues.[14] In mouse embryos, *ALEX2* mRNA is expressed in the developing testis, forebrain and somites, and in dorsal root ganglia and ribs.[16] *In vivo* RNAi screening for potential tumor suppressor genes using immortalized embryonic hepatocytes lacking p53 and overexpressing MYC revealed that knockdown of *ALEX1* and *ALEX2* individually accelerated hepatocarcinogenesis in mice.[17] From these results, the members of the ALEX/ARMCX gene family are suspected to function as a tumor suppressor and a regulatory factor of embryonic development. However, the role and the expression profile of *ALEX1* gene in colorectal tumor are not well examined.

Here we evaluate the effects of *ALEX1* overexpression on colony formation ability of human colorectal carcinoma cell lines and investigate the capability of promoter methylation to silence *ALEX1* gene using no endogenous *ALEX1* mRNA expressing cell lines.

Materials and Methods

Cell culture. HCT116, SW480, and MCF-7 cells were maintained in DMEM (Invitrogen, Tokyo, Japan) and PANC-1 cells were maintained in RPMI1640 (Invitrogen) supplemented with 10% heat-inactivated FBS (Invitrogen) in a 5% CO_2 atmosphere at 37°C.[18]

Conventional RT-PCR. Total RNA from cells was extracted using TRIzol Reagent (Invitrogen) and RNeasy mini kit (Qiagen, Tokyo, Japan), respectively, and then treated with RQ1 RNase-Free DNase (Promega, Tokyo, Japan). First strand cDNA was synthesized from 1 μg of DNase-treated total RNA using Transcriptor Reverse Transcriptase (Roche Diagnostics, Tokyo, Japan) in a total volume of 20 μL reaction mixtures. The PCR reactions contained 20 ng of cDNA, 1× PCR buffer (TaKaRa Bio, Shiga, Japan), 0.2 mM dNTP each, 0.5 units of Ex Taq Polymerase (TaKaRa), and 0.1 μM each primers and were subjected to the following amplification scheme: One cycle at 95°C for 2 min, 25 (for *GAPDH*) or 35 (for *ALEX1*)

[6]To whom correspondence should be addressed.
E-mail: cox2vegf@yahoo.co.jp

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cycles at 95°C for 30 s, 55°C for 1 min, and 72°C for 30 s, and final extension at 72°C for 5 min after the last cycle.

Western blot analysis. Cells were washed once with PBS and then suspended in RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% deoxycholic acid, 1% Nonidet P-40) containing protease inhibitor cocktail (Sigma-Aldrich, Tokyo, Japan). Protein concentrations were quantified using the Quick Start Bradford protein assay (Bio-Rad Laboratories, Tokyo, Japan) according to the manufacturer's instructions. Equal protein level of whole lysates were resolved by SDS-PAGE and transferred onto Immobilon-P transfer membranes (Nihon Millipore, Tokyo, Japan). Anti-human ALEX1/ARMCX1 polyclonel antibody (Abnova, Taipei, Taiwan) and anti-β-actin monoclonal antibody (Abcom, Tokyo, Japan) were used at a dilution of 1:1000 and 1:10 000, respectively.

Bisulfite genomic sequencing. Cells and tissues were digested with 0.1 mg/mL Proteinase K in lysis buffer (10 mM Tris–HCl, pH 7.6, 1 mM EDTA, 100 mM NaCl, 0.3% SDS) at 60°C overnight, and then genomic DNA was purified by the phenol chloroform extraction and ethanol precipitation. Bisulfite treatment of the genomic DNA was carried out with the EpiTect bisulfite kit (Qiagen) according to the manufacturer's instructions. The amplified fragments with the specific primers to the regulatory region, 5′-GTTGGTAAAGAGGAAAATGAGTG-3′ and 5′-CCCTCCCTAATTCAAAACCCT-3′, and exonic CpG island, 5′-GGGTGGAAGAAAGGAATTAGTG-3′ and 5′-TCA-CAATCTCAACCCCAATCTC-3′, of *ALEX1* gene were cloned into the pGEM-T easy vector (Promega), and subsequently sequenced with the BigDye Terminator Cycle Sequencing system (Applied Biosystems). Bisulfite conversion efficiency of non-CpG cytosines were over 98% for all individual clones for each sample.

Plasmid constructs and transfection. Human *ALEX1* gene (accession No. NM_016608) was amplified by PCR and inserted into the *Xho*I site of pCAGIPuro plasmid (kindly provided by Dr H. Niwa, RIKEN), designated as pCAGIPuro/ALEX1. The pCAGIPuro/EGFP plasmid (kindly provided by Dr H. Niwa, RIKEN) encoding enhanced GFP (EGFP) was used as a control. Plasmid transfections were performed by LipofectAMINE 2000 or LipofectAMINE LTX (Invitrogen) according to the manufacturer's instructions.

Colony formation assay. One day before transfection, each cell line was plated in a 24-well plate at 1×10^5 cells/well. After 24 h post-transfection, the cells were replated into 100-mm dishes and cultured in the presence of 5 μg/mL puromycin (Invitrogen, Grand Island, NY, USA) for 4 weeks. The colonies were fixed with methanol, stained with 0.5% crystal violet solution, and counted. All assays were done in triplicate and repeated three times.

Soft agar colony formation assay. A total of 1000 cells of each clone in 1 mL DMEM containing 0.3% agar (Difco Laboratories, Detroit, MI, USA), 10% FBS, and 1 μg/mL puromycin, were plated per well onto six-well plates coated with 1 mL DMEM containing 0.6% agar, 10% FBS, and 1 μg/mL puromycin. After 2 weeks, colonies were stained with 0.5% crystal violet solution and counted. The experiment was performed in triplicate and repeated three times.

Statistical analysis. Statistics were performed using Mann–Whitney's *U*-test. A P-value < 0.05 was considered to be statistically significant.

Results

Overexpression of *ALEX1* suppresses the anchorage-dependent and -independent colony formation of human colorectal carcinoma cell lines. To examine the effect of overexpression of *ALEX1* on cancer cell proliferation, we performed a colony formation assay of two colorectal carcinoma cell lines HCT116 and SW480 and breast carcinoma cell line MCF-7. Quantitative real-time RT-PCR and Western blot analysis showed that *ALEX1* expression was lost in HCT116 and SW480 cells (Fig. 1A,B). In contrast to HCT116 and SW480 cells transfected with control pCAGIPuro/EGFP plasmid successfully formed colonies, those transfected with pCAGIPuro/*ALEX1* plasmid failed to form colonies (Fig. 1C,D), indicating that overexpression of ALEX1 in colorectal carcinoma cells is capable of impairing colony formation. A similar result was observed using MCF-7 cells in which the *ALEX1* protein was endogenously expressed (Fig. 1A,B,E), suggesting that suppression of cancer cell proliferation requires high levels of *ALEX1* protein expression.

To further investigate the role of *ALEX1* in the colorectal carcinoma cell line, we generated stable clones of HCT116 cells expressing *ALEX1* protein, designated as HCT116/*ALEX1*, by limiting dilution after selection in growth medium supplemented with lower concentrations of puromycin (1 μg/mL) than that used in the colony formation assay (5 μg/mL). As control cells, HCT116/empty and HCT116/EGFP clones that express puromycin resistance protein alone and EGFP, respectively, were generated. Western blot analysis showed that *ALEX1* protein was expressed in the four HCT116/*ALEX1* clones, but not in the two HCT116/empty clones and the two HCT116/EGFP clones (Fig. 2A). All stable clones attached to the bottom of the culture dish and grew similarly (data not shown). However, anchorage-independent growth, a hallmark of malignant transformation, was reduced in the four HCT116/*ALEX1* clones (range of average colony number of 37–56) in comparison to the two HCT116/empty and HCT116/EGFP clones (range of average colony number of 120–243 and 170–233, respectively) (Fig. 2B). These findings support the possibility that *ALEX1* functions as a tumor suppressor in colorectal carcinoma cells.

***ALEX1* is silenced by DNA methylation in colorectal carcinoma cell lines.** Several tumor suppressor genes have been shown to be silenced via promoter hypermethylation in numerous colorectal tumors.[19–22] Therefore, DNA methylation is considered to be one mechanism of tumor suppressor gene inactivation, which can function identically to inactivating mutations. Although our previous report demonstrated that the exogenous human *ALEX1* promoter was active in HCT116 and SW480 by luciferase reporter analysis,[18] endogenous *ALEX1* expression was not detected at the mRNA and protein levels in these cells (Fig. 1A,B). These data prompted us to investigate whether the expression of the *ALEX1* gene was silenced by DNA methylation in colorectal carcinoma cell lines. HCT116 and SW480 cells treatment with DNA methyltransferase (DNMT) inhibitor, 5-aza-2′-deoxycytidine (5-aza-dC), for 72 h, resulted in the reactivation of the *ALEX1* gene (Fig. 3A). Furthermore, bisulfite genomic sequencing revealed that the promoter region of the *ALEX1* gene was highly methylated in both HCT116 and SW480 cells in comparison to those in PANC-1 and MCF-7 cells, which express endogenous *ALEX1* mRNA (Figs 1A,3B), whereas an exonic CpG island in the *ALEX1* gene was hypermethylated in all four cell lines regardless of the *ALEX1* mRNA expression levels (Fig. 3B). These results indicate the capability of promoter methylation to silence *ALEX1* gene in HCT116 and SW480 cells.

Discussion

Members of the ARM family of proteins have shown to exert diverse functions, such as signal transduction, cell adhesion, development, and tumorigenesis, through interactions of their ARM repeat domain with several binding partners. Meanwhile, the biological function of members of the *ALEX/ARMCX* family, a novel subgroup of the ARM family, is largely unknown. Here we clearly showed that overexpression of *ALEX1*

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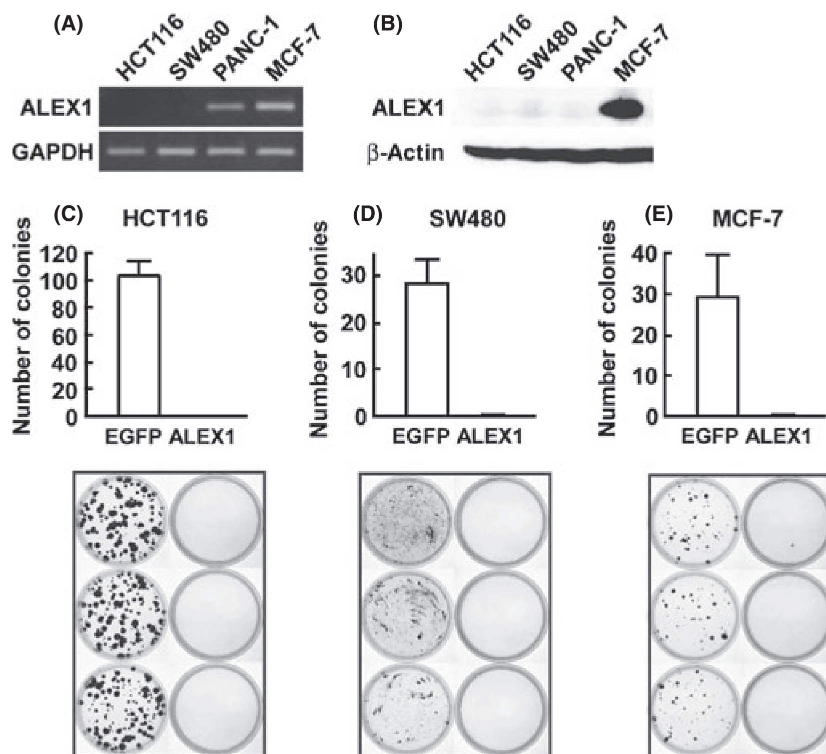

Fig. 1. Effect of *ALEX1* overexpression on the anchorage-dependent colony formation of HCT116, SW480, and MCF-7 cells. (A) Expression of *ALEX1* mRNA in HCT116, SW480, PANC-1 and MCF-7 cells was examined by reverse transcription-polymerase chain reaction (RT-PCR). *GAPDH* mRNA expression was used as an internal control. (B) Western blot analysis for *ALEX1* was performed with cell lysate from HCT116, SW480, PANC-1 and MCF-7 cells. β-Actin protein served as an internal control. Colony formation assay on HCT116 (C), SW480 (D) and MCF-7 (E) cells transfected with pCAGIPuro/enhanced green fluorescent protein (EGFP) or pCAGIPuro/*ALEX1* plasmids, and selected with 5 μg/mL puromycin for 4 weeks. Bar graphs at the upper panel represent the mean number of visible colonies obtained for each cell line from three independent experiments, and error bars represent standard deviation (SD).

suppressed both the anchorage-dependent and -independent colony formation of human colorectal carcinoma cell lines (Figs 1,2). On the other hand, knockdown of *ALEX1* accelerated hepatocarcinogenesis in mice.[17] Together with the reduced expression of *ALEX1* gene in several tumors, these lines of evidence support the hypothesis that *ALEX1* protein functions as a tumor suppressor. Intriguingly, the B variant of specific splicing variant involved in hepatocarcinogenesis (*SVH-B*; also known as armadillo repeat containing 10 [*ARMC10*]), a closely related ARM family member, was identified as an upregulated gene in the human hepatocellular carcinoma by representational difference analysis.[23] The overexpression of SVH-B accelerates cell growth and tumorigenicity in the normal liver cell lines and suppresses the transcriptional activity of tumor suppressor p53.[23,24] Accordingly, *ALEX*-related genes may also be capable of playing different roles in tumorigenesis as well as *APC* and *CTNNB1* function as a tumor suppressor gene and oncogene, respectively.

The first study on *ALEX* family reported that *ALEX1* and *ALEX2* mRNA were decreased in some carcinoma cell lines and tissues. Here we carried out the quantitative expression analysis of the *ALEX1* mRNA in matched tissue pairs of normal colorectal mucosa and colorectal tumor tissues, and showed that the *ALEX1* mRNA was frequently reduced in colorectal tumor. In addition, genome-wide expression profiling of several carcinomas derived from lung, bladder, prostate and uterus using microarray have been accumulated and shown reduction of *ALEX1* mRNA in these cancer tissues.[25,26] Thus, the low or absent *ALEX1* expression seems to be a common feature of a variety of different cancers. It is noteworthy that *ALEX1* was detected at the mRNA level in pancreatic carcinoma cell line PANC-1, but not detected at the protein level, suggesting that posttranscriptional and/or posttranslational mechanisms for *ALEX1* regulation exist.

Aberrant DNA methylation within the promoter of tumor suppressor genes results in the transcriptional silencing of these genes and is believed to contribute to colorectal cancer progression. For example, Wnt antagonists such as secreted frizzled-related proteins, *DICKKOPF-1* and Wnt-inhibitory factor-1, which are the negative feedback regulators of Wnt signaling, are frequently silenced through promoter hypermethylation in colorectal tumors.[19–22] We previously revealed that the expression of *ALEX1* mRNA is upregulated by continuous activation of Wnt/β-catenin signaling.[18] In this study, we demonstrated that the *ALEX1* promoter was hypermethylated and reactivated by DNMT inhibitor in HCT116 and SW480 cells in which Wnt signaling is active, suggesting that DNA methylation serves as one mechanism for the reduction of *ALEX1* gene expression induced by aberrant activation of Wnt signaling although other mechanism(s) such as a downregulation by oncogenic protein may contribute to *ALEX1* gene reduction. Meanwhile, recent reports have indicated that a distinct subset of colorectal cancers showed a high frequency of DNA hypermethylation in multiple genes, which has been termed the CpG island methylator phenotype (CIMP).[27,28] The CIMP has been shown to associate with microsatellite instability and BRAF mutations in serrated

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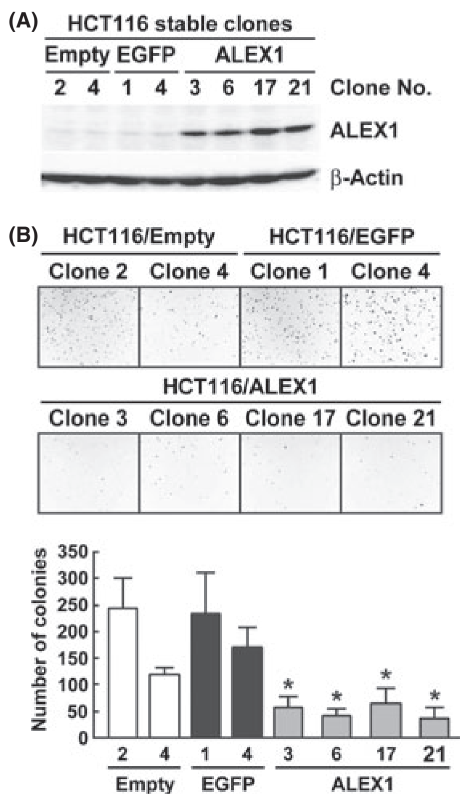

Fig. 2. Effect of *ALEX1* overexpression on the anchorage-independent colony formation of HCT116 cells. (A) Western blot analysis for *ALEX1* was performed with cell lysate from each HCT116/empty, HCT116/enhanced green fluorescent protein (EGFP), and HCT116/*ALEX1* clones. β-Actin protein served as an internal control. (B) Soft-ager colony formation assay with the each stably transfected HCT116 clones. Bar graph at the lower panel represents the mean number of visible colonies obtained for each clones from three independent experiments, and error bars represent SD. $^{*}P < 0.05$ versus each HCT116/empty and HCT116/EGFP clones.

colorectal polyps.[29–32] In our preliminary analysis of DNA methylation status with available five tissue pairs of normal colorectal mucosa and tumor with non-serrated histology from male patients, in which the *ALEX1* mRNA is decreased, the *ALEX1* promoter was hypomethylated in all normal mucosa and four out of five tumor tissues, but hypermethylated in one out of five tumors (data not shown).

Expression of *ALEX1* and *ALEX2* mRNA is lost or significantly reduced in human lung, prostate, colon, pancreas, and ovarian carcinomas and also in cell lines established from different human carcinomas. These genes are, however, normally expressed in cell lines derived from other types of tumors, e.g., sarcomas, neuroblastomas, and gliomas, which will speculate that *ALEX* genes may play a role in suppression of tumors originating from epithelial tissue, i.e., carcinomas. The prognostic significance of *ALEX1* in solid neoplasms has been suggested in several kinds of tumor.[14] We also have examined the correlations between *ALEX1* gene expression in tumor and postoperative prognosis in 49 primary colorectal tumors undergoing complete surgical resection. Patients showing *ALEX1* expressions (n = 17) revealed a significantly better prognosis than those without *ALEX1* (n = 34) (P = 0.045: data not shown). These results possibly imply that silencing of the *ALEX1* gene through promoter hypermethylation possibly leads to the tumor growth in colorectal cancer with the CIMP and Wnt signaling. Therefore examination of *ALEX1* expression might be helpful for predicting the prognosis of patients with curative resected colorectal cancer.

In summary, the current results indicate that overexpression of a non-classical ARM protein family member *ALEX1* suppresses colony formation of human carcinoma cells. Moreover, *ALEX1* was frequently reduced in human colorectal tumor. The possibility that DNA methylation serves as one mechanism for the reduction of *ALEX1* gene expression in human colorectal tumor cell is suggested, although the role of DNA methylation in colorectal tumorigenesis remains to be determined. These findings suggest that *ALEX1* play a negative role in human colorectal tumorigenesis.

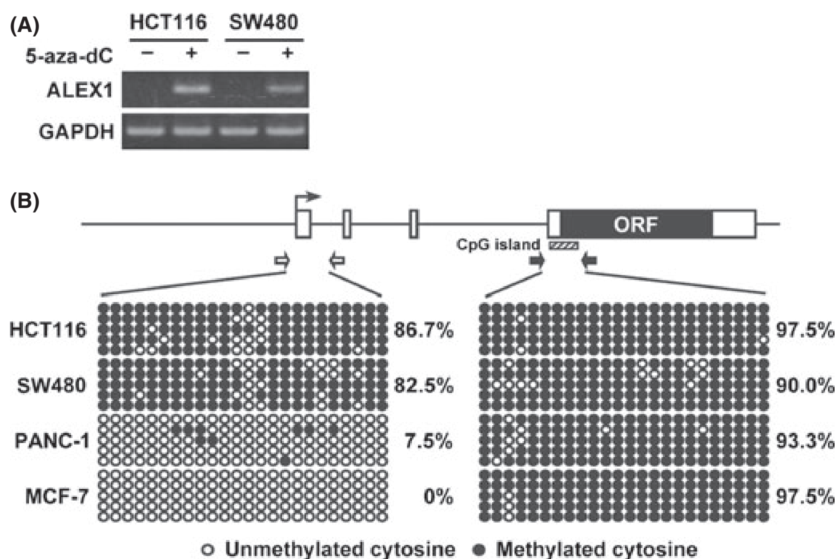

Fig. 3. Silencing of *ALEX1* gene through promoter hypermethylation in HCT116 and SW480 cells. (A) Reverse transcription-polymerase chain reaction (RT-PCR) analysis of *ALEX1* gene in HCT116 and SW480 cells untreated (−) or treated with 0.5 μM 5-aza-dC for 3 days (+). (B) Bisulfite sequencing analysis of *ALEX1* gene in HCT116, SW480, PANC-1 and MCF-7 cell lines. Open and filled boxes represent the exons of the *ALEX1* gene and the open reading frame (ORF) encoding the *ALEX1* protein, respectively. The bent arrow indicates the transcription start site of the *ALEX1* gene. Locations of CpG island (shaded box) and the primers used for promoter region (white arrows) and CpG island (black arrows) are represented above. Open and filled circles represent unmethylated and methylated cytosine, respectively, and each row represents a single clone.

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Disclosure Statement

The authors have no conflict of interest.

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